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Sorghum Ionomics Reveals the Functional *SbHMA3a* Allele That Limits Excess Cadmium Accumulation in Grains

Running head: *SbHMA3a* Limits Sorghum Grain Cd Accumulation

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Abstract

Understanding uptake and redistribution of essential minerals or sequestering of toxic elements is important for optimized crop production. Although the mechanisms controlling mineral transport have been elucidated in rice and other species, little is understood in sorghum—an important C_4 cereal crop. Here, we assessed the genetic factors that govern grain ionome profiles in sorghum using recombinant inbred lines (RILs) derived from a cross between BTx623 and NOG (Takakibi). Pairwise correlation and clustering analysis of 22 elements, measured in sorghum grains harvested under greenhouse conditions, indicated that the parental lines as well as the RILs show different ionomes. In particular, BTx623 accumulated significantly higher levels of cadmium (Cd) than NOG, because of differential root-to-shoot translocation factors between the two lines. Quantitative trait locus (QTL) analysis revealed a prominent QTL for grain Cd concentration on chromosome 2. Detailed analysis identified *SbHMA3a*, encoding a P_{1B}-type ATPase heavy metal transporter, as responsible for low Cd accumulation in grains; the NOG allele encoded a functional HMA3 transporter (SbHMA3a-NOG) whose Cd-transporting activity was confirmed by heterologous expression in yeast. BTx623 possessed a truncated, loss-of-function *SbHMA3a* allele. Functionality of SbHMA3a in NOG was confirmed by Cd concentrations of F_2 grains derived from the reciprocal cross, in which the NOG allele behaved in a dominant manner. We concluded that SbHMA3a-NOG is a Cd transporter that sequesters excess Cd in root tissues, as shown in other HMA3s. Our findings will facilitate isolation of breeding cultivars with low Cd in grains or in exploiting high-Cd cultivars for phytoremediation.

Key words: Cadmium, HMA3 transporter, Ionome, Quantitative trait locus, Recombinant inbred population, Sorghum

Introduction

Plant growth is sustained by balanced homeostasis of essential elements, lack of which can cause growth defects and yield loss in crops (Clemens et al. 2021). As such, plants have developed adaptive strategies to obtain and redistribute sufficient levels of essential elements, while also preventing the accumulation of toxic levels that can have negative effects on plant metabolism (Maathuis 2009). Plants have also evolved mechanisms to circumvent the deleterious effects of toxic elements such as cadmium (Cd) and arsenic (As), which have no known biological function (Williams and Salt 2009). Humans and animals are exposed to these toxic elements predominantly via the food chain leading to adverse health effects (Nawrot et al. 2006). Similar to nutrient elements, the uptake of toxic elements from the soil into plant organs and their efficient movements among plant organs requires the recruitment of different metal transporters, belonging to various transporter families (Sasaki et al. 2016; Ma and Tsay 2021). In rice, for example, As has been shown to be transported via OsABCC1, belonging to the ATP-binding cassette (ABC) transporter subfamily (Song et al. 2014). Zinc (Zn) is transported via the Zinc-regulated, Iron-regulated transporter-like Protein (ZIP) family (Kavitha et al. 2015). This ZIP-family is also shown to transport manganese (Mn), cobalt (Co), copper (Cu), iron (Fe), Cd, and nickel (Ni) (Pedas et al. 2009). In *Arabidopsis thaliana,* several metal transporter families have been identified such as the Cu- and Cd-transporting ATPases, Zn- and Cd-transporting cation diffusion facilitator, cation proton exchangers, which sequester cations such as Mn, lithium (Li), Cd, and calcium (Ca) to the vacuole, and copper transporters (Guerinot 2000; Hirschi et al. 2000; Williams et al. 2000; Kushnir et al. 2001).

The identification of metal transporters occasionally employs ionomics—studies of the ionome—combined with genetic analysis (Doerge 2002; Salt et al. 2008). Such studies have identified numerous quantitative trait loci (QTLs) involved in the control of metal accumulation in crops, especially rice (Huang and Zhao 2020). The transporters responsible for these QTLs have been characterized for Cd as well as Cu and molybdenum (Mo) accumulation (Ueno et al. 2010; Huang et al. 2016; Luo et al. 2018; Yang et al. 2018). Cd is of particular concern because it is highly soluble in water, thus contributing to high toxicity in plants (Qadir et al. 2014). Cd has been shown to cause growth inhibition through various mechanisms such as oxidative stress, alteration of accumulation of other elements, and impairment of photosynthesis (Jing et al. 2005; Liu et al. 2006; Singh et al. 2006). However, many plant species appear to retain up taken Cd in their roots, which protects above-ground tissues from excess Cd exposure and consequently reduces its toxicity (Puig and Peñarrubia 2009; Verbruggen et al. 2009). Mechanisms that actively prevent Cd translocation have been revealed. One such mechanism involves Cd sequestration into the vacuoles by heavy metal ATPase 3 (HMA3) transporters, which have been demonstrated to affect Cd accumulation in major cereal crops including rice, barley, maize, and wheat, as well as *A. thaliana*, and *Brassica rapa* (Morel et al. 2009; Ueno et al. 2010; Wiebe et al. 2010, 2012; Maccaferri et al. 2019; Zhang et al. 2019, 2020; Lei et al. 2020; Tang et

al. 2021). *A. thaliana* HMA3 (AtHMA3) was shown to additionally transport Zn, lead, and Co (Morel et al. 2009).

Despite the focus on Cd toxicity in crops, little is understood about the genetic components involved in Cd accumulation or the overall natural variation in the grain ionome in sorghum. Sorghum is the second most important C_4 cereal crop and is mainly grown for food and fodder (Paterson et al. 2009). It has a relatively small genome size compared with other C_4 grasses, and the reference genome is available (Paterson et al. 2009). Sorghum is also highly syntenic to rice, allowing for the comparison of genetic information between them (Paterson et al. 2009; Ramu et al. 2009). Although several sorghum ionome studies have been reported (Shakoor et al. 2016; Veley et al. 2017; Zhu et al. 2020; Wang et al. 2021), it remains unclear which genetic loci can be exploited for the improvement of grain quality in sorghum. High biomass and sugar index along with tolerance to Cd and Cu make sorghum a suitable crop for phytoremediation, rather than maize and wheat (Bennett and Anex 2009; Calviño and Messing 2012; Metwali et al. 2013). In fact, various studies have focused on the Cd accumulation patterns in sorghum cultivars grown in Cd-contaminated soils, which have shown a wide variation in Cd levels in above-ground tissues. Moreover, the Cd levels in various plant organs were observed to increase with increasing concentrations of exogenous Cd, with the highest levels of Cd being detected in the root tissues and leaf sheaths (Tian et al. 2015; Tsuboi et al. 2017; Jawad Hassan et al. 2020; Liu et al. 2020). While these studies provide a basis for selection of sorghum cultivars for different breeding purposes, sustainable improvement of sorghum quality requires an understanding of the genetic mechanisms involved.

In the present study, we performed grain ionome QTL analysis to investigate genetic loci dictating element accumulation in a recombinant inbred line (RIL) population developed by crossing a US inbred line, BTx623, and a Japanese Takakibi, NOG. Population structure analysis carried out in our previous study demonstrated that BTx623 and NOG are positioned to diverged groups, representing Southern African and Asian accessions, respectively (Kajiya-Kanegae et al. 2020). This RIL population appeared to segregate a broad array of morphological characteristics (Ohnishi et al. 2019; Kajiya-Kanegae et al. 2020; Jing et al. 2021; Takanashi et al. 2021b). Using restriction site-associated DNA sequencing of the F_6 population (213 individuals), we constructed a high-density linkage map consisting of 3,710 single nucleotide polymorphism (SNP) markers, which were shown to identify QTLs responsible for various traits (Kajiya-Kanegae et al. 2020). Our data showed that numerous QTLs were associated with essential and toxic element accumulation in grains, and we focused on a prominent QTL for Cd. Here, we demonstrate that this QTL encodes the functional *SbHMA3a* in NOG, the loss of which resulted in increased translocation of Cd to grains.

Results

Variation in the ionome profile of the parental lines

To estimate whether the RIL population used in this study was a promising resource, we first evaluated the accumulation level of 22 elements in grains of the parental lines, BTx623 and NOG, grown in 2019 and 2020. The ionome of grains harvested in 2019 showed significant variation in 19 out of 22 elements, with NOG accumulating significantly higher levels of 18 elements compared with BTx623, which only accumulated higher levels of Cd. No significant difference was observed in the accumulation of magnesium (Mg), sulfur (S), and Cu (Table 1). In the ionome of grains harvested in 2020, 18 of the 22 elements showed significant differences, whereas 4 elements (boron [B], Mg, phosphorous [P], and germanium [Ge]) showed no differences between the two lines. NOG accumulated significantly higher levels of 11 elements, with the exception of Fe, Ni, Cu, rubidium (Rb), Cd and cesium (Cs), which accumulated more in BTx623 (Table 1). In summary, in both years NOG consistently accumulated significantly higher levels of Li, sodium (Na), potassium (K), Ca, Mn, Co, Zn, As, strontium (Sr), and Mo, whereas BTx623 consistently only accumulated significantly higher levels of Cd (Table 1).

Next, pairwise correlation analysis of elements obtained from five biological replicates in each parental line was conducted to assess the relationships of accumulation among them. In the 2019 data, BTx623 had strong positive correlations among most elements, whereas weak negative correlations were observed between Ge/selenium (Se) and some of the other elements (Fig. 1A). NOG also showed positive correlations among most elements and strong negative correlations were also seen between Ge, Co, Li, and Cd, as well as K, As, and Na (Fig. 1B). In 2020, BTx623 showed strong negative correlations between B, Li, Rb, Cs, Mo, Na, K, and As, as well as Zn, Li, Rb, Cs, and Mo. Weak negative correlations were also observed at a higher frequency compared with in 2019 (Supplementary Figs. S1A). For NOG, larger numbers of strong negative correlations were observed compared with those in 2019. These correlations were observed between Li, Cd, Sr, Ca, P, Se, Zn, K, Cu, As, Cs, Na, and Ni, as well as Mo, Cd, Cu, As, Cs, Na, and Ni (Supplementary Fig. S1B). However, positive correlations between most elements were also observed in both BTx623 and NOG (Supplementary Figs. S1A, S1B). Overall, these results showed the potential genetic diversity of the ionome between the two parental lines, implying their usefulness in conducting QTL analysis of the grain ionome.

Pairwise correlation of elements in the RIL population

For RILs, we subjected 185 individual lines (F_{12}) to ionome analysis, followed by pairwise correlation studies among the elements. The results showed weak positive correlations with two clusters: one between Mo, Fe, Zn, Mg, Cu, P, and S and the other between Ca, Sr, B, and Na (Fig. 1C). F_{13} RILs also showed weak positive correlations between the majority of the elements, with two clusters between Li, B, Mo, Na, Ca, and Sr and the other between Se, Ni, Fe, Cu, Zn, Cd, Ge, Mg, P, and S (Supplementary Fig. S1C). Collectively, the positive correlations and clustering observed among some elements may indicate a shared genetic network, as suggested in a previous study (Karaköy et al. 2012).

Although it has been shown that Cd uptake occurs via transporters for other metals such as Zn, Mn, Fe, and Ca (Clemens 2006), in our results Cd did not show any significant correlation with these elements, especially in the F_{12} RILs (Fig. 1C). This implied that the genetic factors causing variation in Cd accumulation among the RILs were not shared significantly with the mechanisms for the accumulation of other nutrient elements. Based on this, and the parental accumulation of grain Cd, which was consistently higher in BTx623 than in NOG in both growing seasons (Figs. 2A, 2C, Table 1), we focused on the grain Cd accumulation in our RIL population. Although transgressive segregation was observed for Cd in both RIL generations (Figs. 2B, 2D), nearly half of the RILs showed low Cd accumulation similar to NOG. This suggested that there was at least one locus strongly contributing to Cd accumulation, which might be detected in QTL analysis.

Root to shoot translocation of Cd in sorghum seedlings

Root to shoot translocation has been shown as a crucial step influencing the accumulation of Cd in grains (Ueno et al. 2009; Uraguchi et al. 2009). Therefore, we investigated the translocation factors (Cd_{shoot}/Cd_{root}) of the parental lines grown in hydroponic culture where the media included either 1 μM or 3 μM Cd. BTx623 was found to have significantly 2-fold higher shoot Cd levels, compared with NOG in both Cd treatments (Fig. 3A). In contrast, the root Cd concentration was not significantly different (Fig. 3B), although NOG tended to show higher levels. Consequently, BTx623 showed a significantly higher translocation factor, approximately 3-fold higher, than NOG in both Cd treatments (Fig. 3C). Consistent with previous reports, the higher root to shoot Cd translocation in BTx623 appeared to be responsible for the significantly higher Cd concentration in grains than NOG. Seedlings grown in Cd-containing media appeared to show stunted growth in both leaf and root tissues in a dose-dependent manner as a result of Cd toxicity (Figs. 3D-F).

Cd uptake in roots has been suggested to occur through transporters for essential cations such as Zn, Mn, Fe, and Cu (Clemens et al. 1998; Welch and Norvell 1999). To investigate co-accumulation of these cations with Cd, we also evaluated the accumulation of Zn, Mn, Fe, and Cu in roots and shoots of BTx623 and NOG. Significantly higher levels of Zn, Fe, and Cu were observed in the shoots of BTx623 compared with NOG after exposure to 3 μM Cd. Fe and Cu also accumulated significantly higher in BTx623 than NOG at 1 μM Cd treatment. Root metal accumulation showed no significant differences for any of the elements studied (Supplementary Figs. S2A-H). These results suggested that co-accumulation exists between Cd and other cations in BTx623, although not conclusively verified only by this hydroponic experiment.

Ionome QTL analysis

To investigate the genetic loci implicated in variations in element accumulation between BTx623 and NOG grains, we next performed QTL analysis, using genetic markers derived from restriction site-associated DNA sequencing and the ionome data mentioned above. A total of 28 QTLs

were obtained in the F_{12} generation, with logarithm of odds (LOD) scores higher than 3.0 (Fig. 4A, Supplementary Table S1). Each QTL locus was named after the elemental symbol followed by the chromosome number and growing year, such as *qCd2-19*. The phenotypic variances explained by the QTLs ranged from 2.5% to 40.3% (Supplementary Table S1). Overlapping QTLs, defined as QTLs occurring at the same locus, were detected for B and Cd on chromosome 1; Sr and Ca on chromosome 3, Mo, B, and Na on chromosome 4, Se and S on chromosome 7; and P and Fe on chromosome 9 (Fig. 4A). In the F_{13} generation, 22 QTLs were obtained, and phenotypic variances were 3.0% to 18.4% (Supplementary Table S1). Overlapping QTLs were observed for Na, P, and K on chromosome 1; and Ge, Se, Rb, and Mo on chromosome 2; and Sr and B on chromosome 4 (Fig. 4A). Moreover, in both years, QTL clusters of three or more elements found within approximately 30 cM of each other were observed for P, Na, and K on chromosome 1; Mo, Rb, Se, Ge, and Mg on chromosome 2; Sr, B, Na, and Mo on chromosome 4; K, As and Mn on chromosome 5; As, Se, and S on chromosome 7; and Fe, P, and Mn on chromosome 9 (Fig. 4A). Several QTLs were consistently observed in both growing seasons; the QTL for B and Sr on chromosome 4; and Cd on chromosome 2 (Fig. 4A, Supplementary Table S1). Strikingly, *qCd2-19* and *qCd2-20* were prominent, showing the highest LOD scores of 24.5 and 9.5, respectively (Figs. 4B, 4C, Supplementary Table S1). These QTLs also explained a high phenotypic variance of 40.3% and 18.4%, respectively (Supplementary Table S1). Thus, we presumed that *qCd2-19* and *qCd2-20* represent the same locus, *qCd2*. To assess the allelic effect of *qCd2* on Cd accumulation among the RILs, the RILs were divided into two groups, BTx623 and NOG type. Their alleles were determined by their genotypes at the marker with the highest LOD score at *qCd2*, Chr02:8937547. RILs of the NOG type had lower grain Cd concentrations compared with the BTx623 type (Figs. 4D, 4E) indicating that the NOG allele was responsible for low Cd concentrations.

qCd2 fine mapping and phylogenetic analysis of SbHMA3a

Based on the QTL composite interval analysis, *qCd2* was mapped to a region between SNP markers Chr02:8667797 and Chr02:9000127 on chromosome 2 (Fig. 5A, upper panel). In this region, marker density was limited; therefore, we selected more markers to cover the *qCd2* region (Fig. 5A, lower panel). Six RILs showing recombination in the expanded region were found and subjected to further gene mapping (Fig. 5B). As a result, the candidate region was delimited to a 156 kb region flanked by markers Chr02:8857965 and Chr02:9013974, where 17 genes were annotated (Fig. 5B, Supplementary Table S2). Among them, we found two genes, *Sobic.002G083000* and *Sobic.002G083100*, annotated in the database as cation transporting ATPases (https://phytozome-next.jgi.doe.gov/) (Supplementary Table S2). According to this database, OsHMA3 was indicated as one of the protein homologs for both genes. HMA3 transporters have been shown to belong to the P_{1B}-type ATPases, which are involved in Cd transport (Morel et al. 2009; Ueno et al. 2010; Zhang et al. 2019, 2020; Lei et al. 2020; Tang et al. 2021). Therefore, these two genes seemed to be promising candidates of the gene responsible for *qCd2*. Phylogenetic analyses using other HMA proteins from

different plants species including rice, barley, wheat, maize, and *A. thaliana* showed that particularly *Sobic.002G083000,* had close homology with other HMA3 proteins implying that it may similarly function in Cd transport (Supplementary Fig. S3). We termed this gene *SbHMA3a*, in accordance with a previous study where comparative analysis of all HMA genes in rice, maize, and sorghum was performed through database screening (Zhiguo et al. 2018).

Characterization and cloning of SbHMA3a

According to the annotation in the database (derived from BTx623), *SbHMA3a* consists of four exons and three introns, lacking untranslated regions. Compared with NOG sequence data (Accession DRA008159), we found nucleotide polymorphisms that led to three amino acid substitutions (Fig. 6A) within a peptide of 895 amino acids. To verify this annotated transcript, cDNA synthesized from the total RNA of roots of BTx623 and NOG was used as a template to amplify the full-length sequences. Unexpectedly, we found that there was no transcript identical to the annotated one. Instead, an additional 5-bp (TGAAG) existed at the 5' end of the second exon of both BTx623 and NOG (Fig. 6B, Supplementary Fig. S4A). This 5-bp sequence rather derives from the 3' end of the first intron and is likely a result of mis-approximation of the splicing site in the database annotation, resulting in a new gene model. As a result, *SbHMA3a-BTx623* was found to gain a premature termination codon due to a frame shift, resulting in a truncated peptide of 230 amino acids (Fig. 6B, Supplementary Figs. S4B, S4C). However, a 1-bp insertion was observed only in *SbHMA3a-NOG* downstream of the 5-bp addition, thus maintaining an open reading frame almost similar to the annotated one. Additionally, a 6-bp deletion was observed downstream of the 1-bp insertion in NOG, subsequently encoding the original peptide length of 895 amino acids (Fig. 6B, Supplementary Figs. S4A, S4B, S4C). This indel mutation reveals a new *SbHMA3a-NOG* sequence compared to the initial NOG sequence data.

We next tested whether the new gene model identified in our cDNA screening was dominant or other splicing variants exist. A primer pair encompassing the region of variation was used to amplify the corresponding cDNAs by PCR, with root and leaf RNA samples with or without 3 μM Cd (Fig. 6C). No additional bands other than those corresponding to the cDNA clones containing the 5-bp addition were observed (Fig. 6D). Furthermore, direct sequencing of these PCR fragments confirmed that only transcripts with the 5-bp addition at the 5' end of the second exon were detectable (Fig. 6E). Taken together, we concluded that our gene model represents *SbHMA3a*, which implied that BTx623 has a truncated SbHMA3a. Because of such an apparent difference, we further characterized this *SbHMA3a* gene. To examine *SbHMA3a* transcript levels in root and leaf tissues and their response to Cd supplementation, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed with a primer pair spanning the 5-bp addition junction (Fig. 6C). In both BTx623 and NOG, expression of *SbHMA3a* was unaffected by the presence of exogenous Cd. Moreover, the expression levels did not show any obvious difference between BTx623 and NOG. Root tissue accumulated higher levels of *SbHMA3a* compared with leaves, as signals could be observed with as low as 20 PCR cycles

in samples from roots (Fig. 6F). These results suggested that *SbHMA3a* is expressed constitutively, irrespective of exogenous Cd.

Heterologous expression of* SbHMA3a-NOG *in yeast confirming its Cd transporting activity

To evaluate the role of SbHMA3a in Cd transport, constructs carrying *SbHMA3a-BTx623* and *SbHMA3a-NOG* were heterologously expressed in a yeast W△*ycf1* mutant (Uraguchi et al. 2011). *OsHMA3n*, the functional *HMA3* allele derived from rice cv. Nipponbare (Ueno et al. 2010), and empty vector were used as positive and negative controls, respectively. Their expression was driven by a *GAL1* promoter in the pYES2 vector, which is inducible in the presence of galactose (West Jr et al. 1984). In non-inducible glucose media, the growth phenotype was similar among all yeast transformants with different Cd concentrations (Fig. 7A). However, when grown in inducible media containing galactose, yeast cells expressing SbHMA3a-NOG showed hyper-sensitivity to Cd similar to OsHMA3n, whereas cells expressing SbHMA3a-BTx623 and the vector control showed a more tolerant phenotype (Fig. 7B). The growth rate of yeast transformants, grown in liquid media supplemented with galactose and different Cd concentrations, also confirmed the Cd-sensitive phenotype of cells expressing OsHMA3n and SbHMA3a-NOG (Fig. 7C). This increased sensitivity has been suggested to be caused by mis-localization of the proteins to the endoplasmic reticulum rather than the tonoplast in yeast (Ueno et al. 2010; Zhang et al. 2020). Yeast cells expressing SbHMA3a-BTx623 did not show significantly different growth from the vector control except in the presence of 7.5 μM Cd where SbHMA3a-BTx623 was associated with a significantly shorter doubling time (Fig. 7C). These results suggested that SbHMA3a-NOG possesses Cd transport ability, whereas SbHMA3a-BTx623 has little to no transport activity.

Dominant effect of SbHMA3a-NOG on grain Cd accumulation in F_1 and F_2 plants

To examine whether *SbHMA3a-NOG* acts dominantly in accumulating less Cd in grains, we compared Cd concentrations in F_1 and F_2 plants. Assessment of the Cd accumulation in F_2 grains obtained from F_1 plants of reciprocal crosses showed that both F_2-N (NOG as female; i.e. only pollen was provided from BTx623) and F_2-B (BTx623 as female) accumulated significantly lower grain Cd levels compared with BTx623, although their Cd accumulation pattern was similar to that of NOG (Fig. 8A). These results appeared to satisfy our assumption that the NOG allele acts in a dominant manner in limiting the root to shoot translocation of Cd.

To ascertain this dominant effect of the *SbHMA3a* allele, we evaluated Cd accumulation in F_1 shoots and roots. When grown hydroponically, the shoot Cd concentration in F_1-B plants was significantly lower than BTx623 in the presence of exogenous 3 μM Cd, but not different to NOG (Fig. 8B). The root Cd accumulation showed no significant differences between F_1-B and the parental lines (Fig. 8C). Consequently, the root to shoot Cd translocation factor of F_1-B was significantly lower than BTx623 but comparable to NOG (Fig. 8D). These results indicated that the NOG *SbHMA3a* allele indeed contributes to lower Cd accumulation in F_1 shoots. The shoot and root phenotype of seedlings

grown in 3 μM Cd showed that the toxic effects of Cd in F_1-B roots were seemingly similar to that in NOG, further implying the possibility that factors controlling Cd toxicity in NOG were expressed in a dominant manner in F_1 plants (Supplementary Fig. S5). Additionally, we evaluated Cd accumulation in F_1 grains. We assumed that the F_1 grains derived from reciprocal crosses should give contrasting Cd accumulation, and only F_1-N grains should accumulate less Cd. The results indeed showed that F_1-N grains accumulated Cd levels comparable to NOG and significantly lower than BTx623. However, F_1 grains from BTx623xNOG (F_1-B) accumulated significantly lower Cd compared with BTx623 but significantly higher compared with NOG (Fig. 8E). This unexpected accumulation pattern implied that besides maternal influence, pollen from NOG or other factors may also play a role in Cd accumulation during grain filling.

Taken together, the Cd accumulation patterns depicted in the F_1 and F_2 generations led to the conclusion that NOG carries a functional and dominant *SbHMA3a* allele over the BTx623 allele and can complement the null allele of BTx623.

Discussion

Various studies on the sorghum ionome, especially shoot ionome, have been reported previously. For example, Veley et al. (2017) conducted leaf ionome profiling as an indicator of the plant's response to different nitrogen deprivation treatments. Comparative analysis of the sorghum shoot and root ionomes in response to different N, P, and K starvation regimens has also been reported (Zhu et al. 2020). In another study, Wang et al. (2021) focused on high-Cd accumulating sorghum plants by analyzing the correlations between agronomic traits and shoot elemental contents. As for the grain ionome, a genome wide association study of a sorghum association panel grown in different environments has been carried out recently, leading to the detection of numerous SNPs associated with the profile of 20 elements (Shakoor et al. 2016). In this study, we report the full grain ionome profile of a sorghum recombinant inbred population, which led to the identification of 50 QTLs. These can be a good resource to further dissect element accumulation in sorghum.

Ionome QTL mapping has been conducted in many crop species using different mapping populations, such as chromosome segment substitution lines, RILs, backcross inbred lines, genome wide association mapping populations, and multi-parent advanced generation intercross populations (Ishikawa et al. 2005; Norton et al. 2010; Zhang et al. 2014; Pascual et al. 2016; Shakoor et al. 2016). Although the use of each of these populations is associated with its own advantages and shortcomings, the use of biparental populations has been shown to provide a high power for QTL detection and precision in the analysis of rare alleles (Pascual et al. 2016). As indicated by our previous work showing considerable morphological and genetic diversity between NOG and BTx623, our data indicated that the RILs derived from Japanese Takakibi are a useful resource to dissect QTLs controlling the ionome

profile in sorghum, as well as other agronomic traits (Kajiya-Kanegae et al. 2020; Jing et al. 2021; Takanashi et al. 2021b, a). Therefore, we inferred that the high-density genetic map created using this population seems to be sufficient to further elucidate genes responsible for QTLs, as exemplified by *qCd2* leading to the identification of *SbHMA3a* in this study.

Ionome profiling provides insights into the relationships between elements and environmental conditions during different developmental stages in plants (Williams and Salt 2009). The ionome profile of the parental lines used here was observed to vary in each year, which we consider a representation of different environments. For example, in 2019, NOG accumulated significantly higher levels of Fe, Ni, and Rb, than BTx623, whereas in 2020 BTx623 accumulated higher levels (Table 1). Moreover, the grain Cd concentrations increased in both lines in 2020 compared with 2019 (Fig. 2A, 2C). Several possibilities to explain this inconsistency can be drawn; first, different environmental conditions in each year may have resulted in altered soil properties. Second, edaphic factors such as pH and soil density have been shown to affect root and shoot ionome profiles (Jiang et al. 2018), so these effects should be taken into account. In soybean, a varied grain ionome was observed upon application of manure that caused changes in various soil properties (Amiri and Fallahi 2009; Sha et al. 2012). The other possibility is that the sorghum root architecture was inconsistent in each year, further affecting the uptake and redistribution of elements. Baxter (2009) suggested that changes in plant morphology, such as the root structure or developmental stage of the plant, have an impact on the overall plant ionome. The possibility that the mineral element concentrations in the soil varied in the two growing seasons, influencing the root uptake rates, should also be taken into consideration. Indeed, it was suggested that soil minerals can vary even within one field, consequently influencing the plant ionome (Baxter 2009; Wang et al. 2020; Ma and Tsay 2021).

It is noteworthy that significant SNPs associated with candidate genetic loci for the control of grain Zn, Mn, Ni, and Cd levels were identified in sorghum in the study by Shakoor et al. (2016). Although the SNP for Cd they identified was associated with the same candidate gene in our study (*SbHMA3a*), they did not functionally characterize this gene. In this study, characterization of *SbHMA3a* showed that the functional allele is actively involved in limiting the root to shoot translocation of Cd, based on the significantly lower translocation factors observed in NOG compared with BTx623. This was in agreement to previous studies conducted in rice, barley, wheat, *B. rapa*, and *A. thaliana,* showing *HMA3s* as responsible for the control of Cd translocation from roots to above-ground tissues (Morel et al. 2009; Ueno et al. 2010; Zhang et al. 2019, 2020; Lei et al. 2020). The fact that *SbHMA3a* from BTx623 encodes a truncated peptide whereas in NOG it encodes a full-length peptide is the likely cause of the observed variation in translocation factors. In fact, the function of some *BrHMA3* haplotypes was shown to vary depending on whether the haplotype encoded a full-length or truncated peptide (Zhang et al. 2019). Protein topology is an important factor in metal binding capacity (Haque et al. 2022). *HMA3s* belong to the P_{1B}-type ATPases, which carry three hallmark peptide domains, namely the HMA, E1-E2 ATPase, and hydrolase domains. These domains were shown to be

conserved among HMA proteins and necessary for heavy metal transport in plants (Zhiguo et al. 2018; Dabravolski and Isayenkov 2021). Although it is not clearly understood whether each domain can function on its own, our study suggests that expression of the HMA domain alone, as observed in BTx623 (Supplementary Fig. S4B) has little to no function in Cd transport (Fig. 7B, 7C).

Sorghum remains recalcitrant to transformation efforts and only a few studies have been successful (Battraw and Hall 1991; Zhao et al. 2000; Able et al. 2001), although repeatability remains a challenge due to transgene silencing and low transformation frequencies (Azhakanandam and Zhang 2015). In this study, heterologous yeast expression systems were used instead to confirm the Cd-transporting activity of SbHMA3a. Previous studies in rice, *Sedum plumbizincicola*, and *B. rapa*, showed that this yeast heterologous assay can successfully demonstrate the functionality of HMA3s (Ueno et al. 2010; Liu et al. 2017; Zhang et al. 2019, 2020). Consistent with these, our data showed that full-length SbHMA3a-NOG expressed in a W△*ycf1* mutant exhibited Cd transport ability, whereas BTx623 is a loss of function allele (Figs. 7B, 7C). Given that BTx623 has significantly higher shoot and grain Cd concentrations, it was likely that *SbHMA3a* is involved in Cd sequestration into the vacuoles by its localization in tonoplasts, similarly to what was shown in HMA3 orthologues in rice, barley, and maize (Ueno et al. 2010; Lei et al. 2020; Tang et al. 2021).

In addition to vacuolar compartmentalization, plants have also been shown to reduce Cd absorption by precipitating it through secretion of organic acids into the rhizosphere (Nigam et al. 2001). Although translocation of Cd from root to shoot is mitigated by casparian strips in the endodermal layer (Lux et al. 2004), inevitably Cd is taken up in the roots by transporters such as *OsNramp5*, an influx transporter of Cd in rice roots (Ishimaru et al. 2012; Sasaki et al. 2012), and transported to above-ground tissues. *OsHMA2*, a P_{1B}-type ATPase, is involved in this root to shoot transport of Cd (Satoh-Nagasawa et al. 2012; Takahashi et al. 2012; Yamaji et al. 2013). *OsLCT1* likely participates in Cd distribution from the nodes to grains (Uraguchi et al. 2011). Other mechanisms that reduce toxicity include synthesis of chelators or cysteine-rich peptides that bind and detoxify Cd (Luo and Zhang 2021). Plants also cope with Cd toxicity via metal efflux from cells, carried out by efflux transporters. In wheat, *TaTM20* is one such transporter that participates in efflux of Cd from yeast cells, conferring tolerance (Kim et al. 2008). Although our study demonstrated *SbHMA3a* acting as a dominant factor in the two cultivars, whether other factors or genes like *SbHMA3b* contribute to Cd grain concentration remains to be characterized. According to the database annotation, we found that *SbHMA3b* had six exons, five introns and 5' and 3' untranslated regions, and there were four amino acid substitutions in the corresponding NOG sequence, with both alleles encoding 933 amino acids (Supplementary Fig. S6A, S6B). Therefore, the possible role of *SbHMA3b* and its mechanism in Cd transport driven by any of these polymorphisms cannot be ruled out completely.

Based on the presented results, we presume the function of *SbHMA3a,* as summarized in Supplementary Fig. S7. Cd taken up into the root cell is sequestered into the root vacuoles for detoxification, by the functional SbHMA3a resulting in reduced xylem loading and translocation to

above-ground tissues (Supplementary Fig. S7A). On the contrary, non-functional *SbHMA3a* abolishes this function resulting in higher translocation rates through xylem loading and transport to and accumulation in above-ground tissues (Supplementary Fig. S7B). Inferring from this, *SbHMA3a-NOG* should be the dominant allele. Indeed, we confirmed this by analyzing the ionome profile of F_1 and F_2 plants derived from reciprocal crosses (Fig. 8A-D). It is noteworthy that our study revealed that BTx623 has the null allele, contrary to the current annotation. According to the database annotation (https://phytozome-next.jgi.doe.gov/), BTx623 is presumed to carry a functional *SbHMA3a* allele. However, our data on cDNA accumulation *in vivo* and the yeast complementation assay demonstrated that BTx623 carries a null allele due to the presence of an additional 5 bp in the second exon. On the other hand, NOG carries a functional allele owing to an additional indel mutation in the second exon (Fig. 6B). To observe the distribution of these two alleles in other sorghum lines, we conducted *in silico* analysis of *SbHMA3a* from RTx430, BTx642, and Rio available at the Phytozome database (https://phytozome-next.jgi.doe.gov/) and found that RTx430 and Rio have the same *SbHMA3a* haplotype as NOG, whereas BTx642 has the *SbHMA3a-BTx623* haplotype. Given the discovery of the functional allele to reduce Cd concentration in grains, there is potential to exploit the polymorphisms observed in the two haplotypes via marker-assisted selection, in breeding sorghum cultivars with improved grain quality. Analysis of more haplotypes would be of value to reinforce the applicability of these findings.

In conclusion, our study has shown the diversity represented in the ionome profile of two sorghum cultivars; BTx623 and NOG, providing reliable genetic material for further studies on element transport. We showed that *qCd2* encodes *SbHMA3a*, a heavy metal transporter involved in Cd sequestration into the vacuoles. The discoveries made in this study provide useful insights for selection of low Cd genotypes for breeding consumption-safe cultivars, or high Cd genotypes for phytoremediation purposes.

Material and methods

Plant materials and growth conditions

A RIL population, in the F_{12} and F_{13} generations consisting of 185 and 169 RILs respectively, was used. These populations were the progeny of RILs used in our previous study (Kajiya-Kanegae et al. 2020), which was originally generated using a single seed descent method from a cross between BTx623 and NOG (Takakibi). The original seed stock of BTx623 was generously provided by John Mullet and Bill Rooney of Texas A&M University and NOG seeds were purchased from Noguchi seeds (Hannou, Saitama, Japan) and maintained as described by Kajiya-Kanegae et al. (2020). F_1 grains were generated by reciprocal crosses of the two parents. For crossing, open flowers of female plants were removed, and the remaining flowers were emasculated before the onset of anthesis. The emasculated

plants were immediately bagged together with the pollen parent to enhance pollen reception. The cover bags were kept on until grains were uniformly set on the panicles, after which they were removed, and grains were allowed to dry before harvesting.

To obtain grains, 2-week-old sorghum seedlings germinated in a cellular tray were transplanted into pots of 20.5 cm diameter and 18.5 cm depth, with a density of four plants per pot, in a greenhouse with natural day/night conditions at the Institute of Plant Science and Resources (IPSR), latitude: 34° 35' 31'' N, longitude: 133° 46' 7'' E, Kurashiki, Japan. F_2 plants were grown from March to June 2012. F_{12} and F_{13} plants were grown from June to September 2019 and 2020, respectively. Grains were harvested after maturation and dried at 25°C for two weeks then stored at 4°C, to avoid quality deterioration until use.

For hydroponic cultivation, grains of the F_1 generation, BTx623, and NOG were surface sterilized using 5% NaOCl and rinsed three times in water, then transferred to a petri dish layered with a wet paper towel and germinated in an incubator for 2 days at 29°C. They were then transplanted to 2 L plastic boxes containing half-strength Kimura B solution as described previously by Ueno et al., 2009. For Cd treatments, a 10 mM stock solution of $CdCl_2$ (Nacalai Tesque Inc., Japan) was supplemented to final concentrations of 1, 3, 5, and 7 µM as needed. The seedlings were grown for 14 days in a controlled growth environment maintained at 29°C, with a light intensity of 100 µmol/m^2/s and 12-hour day/night cycle. The hydroponic medium was aerated over the entire cultivation period and refreshed every 2 days. Seedlings were harvested and used for subsequent experiments as described below.

Ionome analysis via inductively coupled plasma mass spectrometry (ICP-MS)

Harvested grains were dried for 3 days in a 70°C oven to reduce the moisture content before analysis. Four to five grains were bulked as one sample, and the dry weight was recorded. Harvested shoots and roots of F_1 and parental lines from hydroponic culture were also dried in the same conditions and weighed. Root parts were washed once in 5 mM $CaCl_2$ and rinsed twice with water before drying. Weighed grains, shoots, and roots were digested using 2 mL concentrated HNO_3 followed by addition of 1 mL H_2O_2. The residues were then dissolved in 0.08 M HNO_3 containing 2 µg L^{-1} In as an internal standard. Metal concentration was measured via inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7800).

QTL analysis

For QTL analysis of the ionome data obtained using the F_{12} and F_{13} RILs, a high-density genetic map consisting of 3,710 SNP markers, generated from 213 F_6 RILs in our previous study (Kajiya-Kanegae et al. 2020), was used. All QTL analyses were carried out by composite interval mapping using the R/qtl package as described previously (Broman et al. 2003; Kajiya-Kanegae et al. 2020). A LOD threshold of above three was used to determine a QTL. Pairwise correlation analyses were done

using the R/corr package (Broman et al. 2003). R software version 3.6.1 was used for the QTL and pairwise correlation analyses (Broman et al. 2003).

RNA isolation and cloning of SbHMA3a

Total RNA was extracted from the roots and leaves of BTx623 and NOG seedlings using an RNeasy Plant Mini kit (Qiagen, Germany). Seedlings were prepared using hydroponic culture as described above. First strand cDNA synthesis was performed with 500 ng of RNA using Superscript III™ Reverse Transcriptase (Invitrogen). To clone cDNAs corresponding to *SbHMA3a* (*Sobic.002G083000*), PCR products amplified using cDNA as templates and specific primers, were inserted directly into the high-copy yeast expression vector pYES2. For BTx623 allele, full-length cDNAs were amplified with primers G1-F and G1-R carrying a *Kpn*I site at the 5` and 3`ends and then ligated into the *Kpn*I site of pYES2 using T4 DNA ligase (Takara Bio Inc., Japan). To clone the full-length NOG cDNAs, the same primers and procedures used for BTx623 were employed. Cloned sequences were verified via BigDye™ Terminator v3.1 Cycle Sequencing with appropriate primers. The resultant constructs: pYES2-*SbHMA3a-BTx623* and pYES2-*SbHMA3a-NOG* were used for heterologous yeast expression experiments. Rice *OsHMA3* (*Os07g0232900*) from cv. Nipponbare (*OsHMA3n*) inserted into pYES2 (pYES2-*OsHMA3n*) (Ueno et al. 2010), generously provided by Prof. Jian Feng Ma, was used as a positive control in the heterologous complementation assay.

Gene expression analysis

To conduct semi-quantitative RT-PCR, 500 ng RNA obtained from the roots and leaves of BTx623 and NOG seedlings grown in different Cd concentrations as described above, was used for first strand cDNA synthesis using Superscript III™ Reverse Transcriptase. The cDNA was amplified with primers G1-RT F and G1-RT R to obtain 644 bp fragments. *EIF4a* and *PP2A* were amplified with the primer pairs *EIF4a F* and *EIF4a R* and *PP2A F* and *PP2A R*, respectively, and used as internal controls.

Because different *SbHMA3a* cDNAs were obtained during cloning, we verified the ratio of their abundance in root and leaf cDNA pools by conducting semi-quantitative RT-PCR using primers G1-F and G1-RT R. These primers flank the first intron (as shown in Fig. 6C), which was the region of variation among the identified transcripts. Cloned cDNAs of the 5-bp addition transcripts (Fig. 6B) and genomic DNA were also used as control templates. Primer sequences used are listed in Supplementary Table S3.

Phylogenetic analysis of SbHMA3a

Nucleotide sequences of *SbHMA3a* of BTx623 and NOG were translated into amino-acid sequences using Emboss Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). The amino acid sequences were multiple-aligned with other HMA proteins from rice, *A. thaliana,* sorghum, maize, wheat, and barley using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/), and the phylogenetic

tree was constructed using MEGAX with 1,000 bootstrap replicates (Kumar et al. 2018). Gene structures were predicted using Gene Structure Display (http://gsds.cbi.pku.edu.cn/), peptide domains were predicted using SMART (http://smart.embl-heidelberg.de/), and alignment of the BTx623 and NOG SbHMA3a peptide sequences was done using Boxshade (https://embnet.vital-it.ch/software/BOX_form.html). Accession numbers of the HMA proteins are listed in Supplementary Table S4.

Yeast heterologous complementation assay

To assess the Cd transport activity of SbHMA3a, pYES2-*SbHMA3a-BTx623*, pYES2-*SbHMA3a-NOG*, and pYES2-*OsHMA3n* (rice gene as a positive control) constructed as described above were used in a yeast heterologous assay. The empty vector pYES2 as a negative control, and the three constructs (100 ng each), were used to transform a Cd-sensitive mutant, W△*ycf1* (Uraguchi et al. 2011) using the lithium acetate transformation method (Gietz et al. 1995). Positive transformants were selected on solid media containing 2% (w/v) glucose, 6.7 g L^{-1} yeast nitrogen base without amino acids (Sigma, St Louis, MO, USA), 1.92 g L^{-1} yeast synthetic dropout medium without uracil (Sigma, St Louis, MO, USA) (SD-Ura), 2% agar, and 300 mg mL^{-1} hygromycin. For the spotting assays, the transformants were grown at 30°C to mid-exponential phase in SD-Ura liquid media. The OD_{600} was adjusted to 1, and four 1:10 serial dilutions were then spotted on SD-Ura solid media replacing glucose with 2% (w/v) galactose, supplemented with either 0, 20, 30, or 40 μM $CdCl_2$. Yeast was incubated for 3 days at 30°C.

For quantitative evaluation of the growth rate of the recombinant yeast cells, their doubling time was examined. From a starting OD_{600} of 0.2, OD_{600} values were measured at ten time points within a 30-hour growth period at 28°C in liquid SD-Ura media containing 2% (w/v) galactose and different Cd concentrations (0, 2.5, 5, 7.5, and 10 μM $CdCl_2$). The growth rate of the cells was calculated using the exponential equation $y=Ae^{Bx}$, where: y is the number of cells at any given time point, A is the initial amount of cells, e is a constant, B is the growth rate and x is time (hours). Doubling time, which is the time taken for cells to double in number, was then calculated as doubling time (T_d) = ln(2)/B. The experiments were done in three biological replicates.

Statistical analysis

Data were analyzed using Student's *t*-test to compare elemental concentrations in grains, shoots, and roots. For other statistical analyses, one-way ANOVA was done followed by comparisons of means using Tukey test or Dunnett's test. Significant differences were defined as $P < 0.05$.

Data Availability Statement

The NOG reference sequence data used in this paper appear at the DDBJ Sequence Read Archive with the accession number DRA008159. *SbHMA3a-NOG* and *SbHMA3a-BTx623* cDNA

sequence data revealed by this study are available at the DDBJ Sequence Read Archive. The ionome data underlying this article are not deposited to the publica domain but will be shared on reasonable request to the corresponding author.

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Author Contributions

W.S. conceived the work analyzing sorghum ionome along with K.Y. and T.F.; material preparation, field experiments, and data analysis of QTLs were performed by F.W.W., K.Y., Z.J., T.T. H.T., T.K., and H.K.-K., with supervision by W.S., T.F, H.I., and N.T.; RILs were established by W.S and prepared by F.W.W.; final data were prepared for publication by F.W.W., K.Y., H.T., and W.S., and the manuscript was written by F.W.W., and W.S., on behalf of all authors.

Disclosures

The authors have no conflicts of interest to declare.

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Table 1 Comparison of the grain ionome in BTx623 and NOG in 2019 and 2020.

Elemental concentrations in grains (mg/kg DW)				
	2019		**2020**	
Element	BTx623	NOG	BTx623	NOG
Li	0.0002 ± 0.0002	0.008 ± 0.001 ***	0.005 ± 0.0004	0.022 ± 0.001 ***
B	0.697 ± 0.09	0.953 ± 0.1 *	1.163 ± 0.1	1.376 ± 0.2 NS
Na	13.736 ± 1.5	19.803 ± 1.4 ***	13.046 ± 1.2	22.487 ± 3.6 **
Mg	1537.22 ± 108	1563.76 ± 98.4 NS	1647.23 ± 103.4	1638.84 ± 128 NS
P	3654.46 ± 155.1	4151.71 ± 111.1 ***	4048.21 ± 119.7	4461.75 ± 345.7 NS
S	1089.28 ± 53	1136.49 ± 68.1 NS	1172.89 ± 65.3	1473.09 ± 106.3 **
K	3567.18 ± 169.1	4658.42 ± 253.5 ***	4046.46 ± 138.4	4999.28 ± 326.7 **
Ca	81.103 ± 9	119.14 ± 12.6 ***	75.44 ± 5.8	166.23 ± 26.6 **
Mn	16.404 ± 1	31.42 ± 2.4 ***	30.699 ± 2.06	37.644 ± 2.5 **
Fe	30.34 ± 1.7	33.82 ± 1.9 *	45.27 ± 3.5 **	32.57 ± 6.2
Co	0.003 ± 0.0003	0.02 ± 0.004 ***	0.021 ± 0.001	0.032 ± 0.002 ***
Ni	0.24 ± 0.016	0.81 ± 0.1 **	0.796 ± 0.08 **	0.617 ± 0.05
Cu	5.873 ± 0.6	6.498 ± 0.5 NS	9.425 ± 1.3 **	6.479 ± 1.3
Zn	21.568 ± 1.4	34.598 ± 2.8 ***	31.096 ± 3.5	45.219 ± 5.1 **
Ge	0.0021 ± 0.0003	0.0025 ± 0.0002 *	0.0029 ± 0.0002	0.0027 ± 0.0004 NS
As	0.01 ± 0.001	0.027 ± 0.002 ***	0.033 ± 0.002	0.08 ± 0.01 **
Se	0.012 ± 0.003	0.02 ± 0.003 ***	0.014 ± 0.001	0.02 ± 0.01 **NS**
Rb	1.991 ± 0.093	4.317 ± 0.2 ***	4.732 ± 0.2 ***	1.529 ± 0.06
Sr	0.169 ± 0.021	0.208 ± 0.02 *	0.115 ± 0.01	0.303 ± 0.03 ***
Mo	0.631 ± 0.048	0.972 ± 0.1 **	0.339 ± 0.02	1.562 ± 0.1 ***
Cd	0.128 ± 0.017 ***	0.007 ± 0.001	0.182 ± 0.02 ***	0.082 ± 0.01
Cs	0.002 ± 0.0001	0.009 ± 0.001 ***	0.015 ± 0.0007 ***	0.003 ± 0.0007

*, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively, and NS shows no significant difference, calculated using Student's *t*-test. Data are means ± SD of 5 biological replicates.

Figure legends

Figure 1

Correlation matrix showing associations between the elements measured in the 2019 growing season. The elements were analyzed from grains and pairwise correlations conducted for (A) data obtained from 5 biological replicates of BTx623, (B) data obtained from 5 biological replicates of NOG, and (C) 185 F_{12} RILs, using Pearson's correlation coefficients. Deep blue and deep red colors denote strong positive and negative correlation between elements, respectively. The size of the circles is proportionate to the strength of correlation.

Figure 2

Grain Cd concentration of parental lines, and frequency distributions of RILs. (A) Grain Cd concentration in parental lines harvested in 2019 and (B) the frequency distribution of F_{12} RILs. (C) Grain Cd concentration in parental lines harvested in 2020 and (D) the frequency distribution of F_{13} RILs. Parental mean values are indicated by orange arrows for NOG and blue arrows for BTx623. Data in (A) and (C) are means ± SD of 5 biological replicates. DW represents grain dry weight. Asterisks indicate $P < 0.001$, calculated using Student's *t*-test.

Figure 3

Cd accumulation and phenotypes in shoots and roots of parental lines grown in a range of Cd concentrations. BTx623 and NOG lines were grown in hydroponic conditions with different Cd concentrations for 14 days. The levels of Cd accumulation in (A) shoots and (B) roots of the two lines were compared. (C) Cd translocation factor was calculated as the ratio of shoot/root concentrations. (D-F) The phenotype of seedlings grown in the absence or presence of 1 μM and 3 μM Cd for 14 days. Scale bars indicate 5 cm. Data in (A), (B), and (C) are means ± SD of 3 biological replicates. * and ** denote significant differences at $P < 0.05$ and $P < 0.01$ respectively, calculated using Student's *t*-test, NS indicates not significant, DW grain dry weight.

Figure 4

QTL analysis of grain metal concentrations in the F_{12} and F_{13} RIL populations. (A) Map of QTLs obtained in the two generations spread across the 10 chromosomes of sorghum. The position of transcriptional start point for *SbHMA3a* is shown in magenta on the left side of chromosome 2. (B) and (C) The logarithm of odds (LOD) graphs showing a prominent QTL obtained for Cd (*qCd2*) on chromosome 2 with LOD scores of 24.5 and 9.5 using the F_{12} and F_{13} RIL population data, respectively. The gray line represents a LOD threshold of 3, whereas the red line is a LOD threshold based on a permutation test with 1,000 iterations. (D) and (E) Plots showing the grain Cd concentration of the F_{12} and F_{13} RILs, respectively. The RILs were divided into two groups, BTx623 and NOG type, depending

on their genotype at the marker with highest LOD score at *qCd2.* The mean values of Cd concentration are denoted by blue and red lines. DW represents grain dry weight.

Figure 5

Fine mapping of *qCd2* on chromosome 2. Fine mapping of *qCd2* was done using graphical genotypes of selected recombinants. (A) *qCd2* was fine mapped using 16 markers flanking the main marker, Chr02:8937547, to the right and left sides. (B) RILs showing recombination in this target region and with varying grain Cd concentrations were selected. The candidate region was delimited to a 156 kb interval between markers Chr02:8857965 and Chr02:9013974. Black and white segments show homozygous BTx623 and homozygous NOG genotypes, respectively. The corresponding Cd concentration of each recombinant is indicated on the right side.

Figure 6

Schematic representation of SbHMA3a gene structures obtained from a database and cDNA sequencing, detection of a major SbHMA3a transcript and semi-quantitative RT-PCR analysis. (A) The gene structure of *SbHMA3a* of NOG was constructed with that of BTx623 annotated in the Phytozome v13 database (https://phytozome-next.jgi.doe.gov/). Base and amino acid substitutions in the NOG sequence are shown. (B) The gene structures of cloned *SbHMA3a* of BTx623 and NOG were obtained from the results of cDNA sequencing. Both cDNA sequences showed a 5-bp addition immediately upstream of exon 2 (gray bar), which introduced a frameshift and premature termination codon (stop gain) in the second exon of *SbHMA3a-BTx623*. *SbHMA3a-NOG* contains a 1-bp insertion and 6-bp deletion just after the frameshift so that the rest of the sequence remains in frame and maintains its original length. Black bars represent exons, and blue hats represent introns. (C) *SbHMA3a* transcript abundance in a cDNA pool was evaluated by RT-PCR using primers flanking the variable region on the first intron. F1 and R represent the forward (G1 F) and reverse (G1-RT R) primers, respectively. (D) The PCR amplicons were analyzed on a 4% agarose gel. cDNAs were synthesized from roots and leaves of seedlings grown in the absence (-) or presence (+) of 3 μM Cd. Genomic DNA and cloned plasmid DNA were used as controls. (E) Wave data and sequences of PCR amplicons in (D), showing the detection of 5-bp addition in the second exon of both BTx623 and NOG alleles of *SbHMA3a*, indicated in black boxes. (F) Comparison of *SbHMA3a* transcript accumulation in the roots and leaves of BTx623 and NOG at different Cd concentrations (0, 1, 3, 5, and 7 μM) and PCR cycles. F2 and R in (C) represent the forward (G1-RT F) and reverse (G1-RT R) primers used, respectively. *EIFa* and *PP2A* genes were used as internal controls in roots and leaves respectively.

Figure 7

Functional assay of SbHMA3a in yeast. *Saccharomyces cerevisiae* mutant *ycf1* was transformed with plasmids carrying *SbHMA3a-BTx623*, *SbHMA3a-NOG*, and empty vector (negative control), under the

control of a GAL1 promoter. *OsHMA3n* was used as a positive control. (A) Cells (OD_{600} of 1) were spotted in four 10-fold serial dilutions on plates containing either glucose or (B) galactose, and varying Cd concentrations. (C) Cells (OD_{600} of 0.2) were grown with shaking in liquid SD-Ura media supplemented with galactose (2% w/v) and different Cd concentrations (0 to 10 μM Cd) for 30 hours at 28°C. Cell doubling time (hrs) was calculated as described in materials and methods. Different letters indicate significant differences in each Cd treatment calculated using Tukey's test ($P < 0.05$). Data are means ± SD of 3 biological replicates.

Figure 8

Cd accumulation in F_1 and F_2 generations. Reciprocal crosses were made between parental lines to evaluate the Cd accumulation patterns in grains and shoots. (A) Cd concentration in the grains of F_2 reciprocal crosses and parental lines. (B) Shoot and (C) root Cd concentrations of F_1 plants and parental lines. (D) Cd translocation factors calculated as the ratio of shoot/root concentrations. (E) Cd concentrations in the grains of F_1 reciprocal crosses and parental lines. Different letters denote significant differences calculated using Tukey's test at $P < 0.05$ in panel (A) and (E), and asterisks in (B)-(D) indicate significant difference at $P < 0.05$ calculated using Dunnett's test. Data are means ± SD of 3 biological replicates.